A MOUSE β-GLOBIN MUTANT THAT IS AN EXACT MODEL OF HEMOGLOBIN RAINIER IN MAN

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ABSTRACT

A mutation induced by ethylnitrosourea in a spermatogonial stem cell of a 101/H mouse has resulted in a structurally altered β-diffuse major globin in one of his offspring. The mutant hemoglobin is associated with polycythemia, rubor, increased oxygen affinity and decreased hem-hem interaction. The mutant haplotype has been designated Hbb^{d4}, polycythemia. Amino acid analysis of the mutant globin has shown that a single substitution β145 Tyr → Cys has occurred, and it is proposed that ethylnitrosourea induced an A → G transition in the tyrosine codon (TAC → TGC). This murine polycythemia is homologous with hemoglobin Rainier in man, in which the amino acid substitution is also β145 Tyr → Cys and which is associated with similar physiological consequences.

MORE than 400 structurally altered hemoglobins have been described in man, and more than 250 of these are due to changes in the β-globin polypeptide (MCKUSICK 1983). Most of these have single amino acid replacements, attributable to single-base changes in the DNA of the globin genes. In some instances the substitution of one amino acid by another results in a hemoglobin with altered properties, which may lead to a hemoglobinopathy such as anemia, cyanosis or polycythemia.

By contrast, in the mouse, relatively few structurally altered hemoglobins are known. In adult mice two β-globin and two α-globin genes are expressed (HILSE and POPP 1968; LEDER *et al.* 1981; KONKEL, MAIZEL and LEDER 1979; WEAVER *et al.* 1979). Polymorphism of *Hbb* (hemoglobin β-chain) is widespread in natural populations (BERRY 1978) and three haplotypes, Hbb^d, Hbb^s and Hbb^p, have been described, which are also found in inbred strains (RUSSELL and MCFARLAND 1974). In those strains that are Hbb^s one type of β-globin chain is synthesized in the adult, βsingle, whereas in those that are Hbb^d or Hbb^p two β-globin polypeptides are found in unequal amounts. In both Hbb^d and Hbb^p 80% of the adult β-globin is in the form β-diffuse major (βdmaj). The remaining 20% in Hbb^d occurs as β-diffuse minor (βdmin), but in Hbb^p mice a variant β-minor polypeptide, β-peculiar minor (βpmin) occurs (RUSSELL and MCFARLAND 1974). The amino acid sequence of the four different types of adult β-globin chain, βsingle, βdmaj, βdmin, and βpmin, is known (POPP 1973; POPP and BAILIFF 1973; GILMAN 1976a,b). The βsingle and βdmaj

chains are very similar and only differ at three of the 146 amino acids in the β-globin polypeptide. There are nine amino acid substitutions between βdmin and βdmaj and 11 between βdmin and βsingle, and βpmin differs from βdmin at two additional positions. Hemoglobinopathy is not associated with any of these *Hbb* haplotypes, although there is evidence from natural populations that genotypic or allelic frequencies at *Hbb* can alter in response to changing selective pressures (BERRY 1978).

One other naturally occurring *Hbb* haplotype, Hbb^{th-1}, is known (SKOW *et al.* 1983) which leads to β-thalassemia. Hbb^{th-1} causes deficiency of normal β-globin polypeptides and results from deletion of about 3.3 kb of DNA, including regulatory sequences and all coding blocks for βdmaj globin (SKOW *et al.* 1983). Two mutants affecting expression of β-globin genes have been induced by X rays; one was the result of tandem duplication of a segment of chromosome 7 which included the β-globin loci (RUSSELL *et al.* 1976) and the other arose by nondisjunction of chromosome 7 (RUSSELL *et al.* 1976). The chemical ethylnitrosourea (ENU) has induced an electrophoretric mobility change in the βdmin globin chain (MUROTA, SHIBUYA and TUTIKAWA 1982).

We describe here a structurally altered hemoglobin in mice in which an amino acid substitution has occurred in the βdmaj chain. This replacement has arisen as a result of a mutation induced by the alkylating agent, ENU. The amino acid alteration has resulted in a hemoglobin with a high affinity for oxygen, and, as a consequence, mice carrying this mutation are polycythemic. The mutation appears to be homologous with that found in hemoglobin Rainier in man (ADAMSON, PARER and STAMATOYANNOPOULOS 1969).

MATERIALS AND METHODS

Mice of the inbred strains BALB/c and 101/H, and the outbred mutation testing stock, T, were used. Male 101/H mice between 9 and 12 wk of age were given an intraperitoneal injection of ENU at a dose of 250 mg/kg. ENU (East Anglian Chemicals Ltd.) was dissolved in 0.2 M phosphate/citrate buffer as described by RUSSELL *et al.* (1979). All injections were completed within 1 hr of dissolving ENU in the buffer. Two months after treatment the males were mated to T stock females selected to be Hbb^s/Hbb^s and Hba^h/Hba^h. Thus, any mutations detected would have arisen in spermatogonial stem cells. The (T × 101/H)F_1 male in which the mutation arose was backcrossed to both 101/H and T stock females, and from these backcrosses the respeective heterozygotes Hbb^d/Hbb^{d4} and Hbb^s/Hbb^{d4} were selected and intercrossed to obtain Hbb^{d4}/Hbb^{d4} homozygotes.

For analysis of hemoglobin α-chain (*Hba*) and hemoglobin β-chain (*Hbb*) 50 μl of blood were taken from (T × 101/H)F_1 offspring and other mice at 4 wk of age or older by retroorbital bleeding into heparinized microhematocrit tubes. Fifty microliters of distilled water were added to 5 μl of the blood sample and HBA phenotypes were resolved by isoelectric focusing of this diluted lysate as described by WHITNEY *et al.* (1979). The remainder of the blood was separated into red cell and plasma components, and the red cells were taken for cellulose acetate electrophoresis on Helena Titan III plates to ascertain HBB phenotypes (WHITNEY 1978). To examine HBB in 14-day embryos, blood was collected and red cells were separated as described by WHITNEY and RUSSELL (1980). One microliter of embryonic red cells was lysed in 20 μl of cystamine, and the lysate was used for cellulose acetate electrophoresis (WHITNEY 1978).

Oxygen dissociation curves were determined as described by NEWTON and PETERS (1983).

Hematological observations were made on blood, obtained by phlebotomy of the tail and appropriately diluted for counting red corpuscles and nucleated cells; blood was heparinized for determination of microhematocrit and was hemolyzed in ammoniated water for determination of

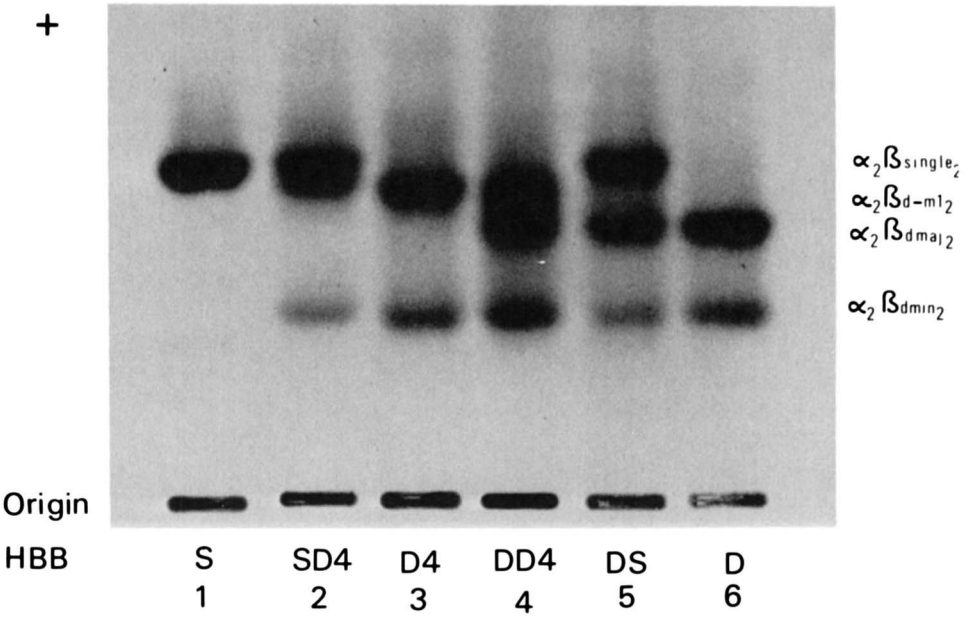

FIGURE 1.—Photograph to show HBB in cystamine-treated hemolysates from mice of the polycythemic stocks after cellulose acetate electrophoresis. The subunit composition of the bands is shown on the right.

hemoglobin (MRC gray wedge photometer). Mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were obtained by calculation.

For amino acid analysis mouse globin chains were fractionated by a modification of the method of CLEGG, NAUGHTON and WEATHERALL (1966). Isolated β-chains were aminoethylated and digested with trypsin, and the tryptic peptides were fractionated by two-dimensional paper electrophoresis and chromatography. Peptides were located by ninhydrin staining, eluted in 50 μl of 6 N HCl and hydrolyzed for 16 hr at 105° in sealed capillaries, and the amino acid compositions were determined on a Beckman 119 CL analyzer.

RESULTS

Electrophoresis: The mutation was found during electrophoretic screening for induced mutations in (T × 101/H)F_1 progeny of T stock females and 101/H males treated with 250 mg/kg of ENU. Figure 1 is a photograph of part of a cellulose acetate plate showing the electrophoretic separation of cystamine-treated hemoglobin. The T stock has the *Hbb*^{s} haplotype, and the HBB-S phenotype consists of one hemoglobin band, $\alpha_2\beta$single$_2$ (channel 1). A weak component cathodal to $\alpha_2\beta$dmin$_2$ may also be seen; this weak band is only visible after staining the plate for protein (Ponceau S in this instance) and is carbonic anhydrase. The 101/H males have the *Hbb*^{d} haplotype, and the HBB-D phenotype on this system is made up of two bands of subunit composition $\alpha_2\beta$dmaj$_2$ and $\alpha_2\beta$dmin$_2$ (channel 6). In the absence of mutation, (T × 101/H)F_1 are expected to have the HBB-DS phenotype (channel 5) which is a mixture of the two parental phenotypes. During the course of the experiment one male (T × 101/H)F_1 was found with the HBB-SD4 phenotype (channel 2)

in which $\alpha_2\beta$dmin$_2$ is present but $\alpha_2\beta$dmaj$_2$ is missing and $\alpha_2\beta$single$_2$ appears broader than in either HBB-DS or HBB-S. Subsequent electrophoretic analysis of the hemoglobin of both parents showed that they had the phenotypes expected, HBB-S for the T stock mother and HBB-D for the 101/H father. All other progeny from this mating were HBB-DS. Thus, the mutation appeared to have been induced by ENU in the 101/H male. When the HBB-SD4 male was backcrossed to 101/H females (HBB-D × HBB-SD4) two sorts of progeny were found, those with the HBB-SD4 phenotype like the male parent and those with a new phenotype HBB-DD4 (channel 4) in which three bands can be seen, $\alpha_2\beta$dmin$_2$, $\alpha_2\beta$dmaj$_2$ and a new band, called $\alpha_2\beta$d-m1$_2$, of greater anodal electrophoretic mobility. When mice of phenotype HBB-DD4 were intercrossed, three sorts of progeny were found, HBB-D, HBB-DD4 and HBB-D4 (channel 3). In HBB-D4, two bands, $\alpha_2\beta$dmin$_2$ and $\alpha_2\beta$d-m1$_2$, are present. The simplest interpretation of these patterns is that a mutation arose in a germ cell of the original 101/H male affecting the mobility of the $\alpha_2\beta$dmaj$_2$ hemoglobin and that the original mutant mouse was heterozygous for this mutant allele. Thus, the broad anodal band seen in HBB-SD4 is composed of both $\alpha_2\beta$single$_2$ and $\alpha_2\beta$d-m1$_2$. Thus, mice of phenotype HBB-DD4 are heterozygous Hbb^{d}/Hbb^{d4} and HBB-D4 mice are homozygotes Hbb^{d4}/Hbb^{d4}.

The alteration in electrophoretic mobility was only seen in cystamine-treated samples. When samples were treated with 3 mM EDTA or with 1 mM dithiothreitol, hemolysates from Hbb^{d}/Hbb^{d} and Hbb^{d4}/Hbb^{d4} mice had an identical appearance on electrophoresis.

Because the electrophoretic separation was carried out on intact hemoglobin and not on isolated β-globin or α-globin chains, it was possible that the mutational event had occurred at *Hba* (which codes for α-globin), not *Hbb*. Linkage analysis using data from two different crosses confirmed that the mutation was located on chromosome 7 in the *Hbb* region (Table 1). Mice of the T stock carry chinchilla (c^{ch}) an allele of albino (c) on chromosome 7. In the first cross the original mutant male of presumed genotype $c^{ch}Hbb^{d}/+Hbb^{d4}$ was backcrossed to a T stock female of genotype $c^{ch}Hbb^{d}/c^{ch}Hbb^{d}$, and 15 backcross progeny were classified for c^{ch} and HBB (Table 1). In the second cross two BALB/c females ($cHbb^{d}/cHbb^{d}$) were crossed to a male of the mutant stock ($+Hbb^{d4}/+Hbb^{d}$), and F_1 females that were $cHbb^{d}/+Hbb^{d4}$ were selected and backcrossed to BALB/c males. Only those backcross progeny that were c/c were typed for HBB since an additional aim of this experiment was to commence the transfer of Hbb^{d4} onto a c/c background. Forty progeny were typed for HBB (Table 1). The data show linkage between c or c^{ch} and Hbb^{d4}. The recombination frequency was in agreement with previous data (DAVISSON and RODERICK 1981). Thus, it seems reasonable to assume that the mutation had occurred at *Hbb*.

Hematology: Data on tail blood are presented in Table 2. The homozygote, Hbb^{d4}/Hbb^{d4}, had significantly elevated red blood cell counts, hematocrit values and hemoglobin concentrations and somewhat increased MCV (normal values 45–50 μm^3) compared to both heterozygous (Hbb^{d}/Hbb^{d4} and Hbb^{s}/Hbb^{d4}) and homozygous normal (Hbb^{d}/Hbb^{d} and Hbb^{s}/Hbb^{s}) litter mates. In addition, the

TABLE 1

Linkage between c *or* c^{ch} *and* Hbb^{d4}

Parental mating	Progeny class	Progeny genotype		No. from heterozygous	
		c/c^{ch}	*Hbb*	Female	Male
Cross 1:					
$c^{ch}Hbb^{s}/c^{ch}Hbb^{s}$	Parental or nonrecombi-	c^{ch}/c^{ch}	*s/s*		7
×	nant	$c^{ch}/+$	*s/d4*		7
$c^{ch}Hbb^{s}/+Hbb^{d4}$	Recombinant	c^{ch}/c^{ch}	*s/d4*		1
	c^{ch}-*Hbb*	$c^{ch}/+$	*s/s*		0
					15
Cross 2:					
$c\ Hbb^{d}/+Hbb^{d4}$	Parental or nonrecombi-	*c/c*	*d/d*	36	
×	nant				
$c\ Hbb^{d}/c\ Hbb^{d}$	Recombinant	*c/c*	*d4/d*	4	
	c-Hbb			40	

Percentage recombination using combined data from cross 1 and cross 2 = 9.1 ± 3.9.

ears, feet and tails of Hbb^{d4}/Hbb^{d4} mice were markedly more pink in color than those of heterozygotes or normal homozygotes. Significantly elevated values for red cell number, hematocrit and hemoglobin concentration were found in heterozygotes when compared with homozygous normal littermates, but the elevations were less marked than those found for the homozygote Hbb^{d4}/Hbb^{d4} and the mean cell volume was normal. Mean cell hemoglobin concentration and nucleated cell counts were within normal values for both the polycythemic homozygote and the heterozygotes. Red cells were of normal morphology, but staining with brilliant cresyl blue revealed Heinz bodies. Thus, both the heterozygote and homozygote, Hbb^{d4}/Hbb^{d4}, are polycythemic and have increased red cell masses.

Inheritance: Inheritance of Hbb^{d4} was investigated by examining HBB in offspring of at least 4 wk of age from backcrosses and intercrosses. Two classes of progeny were found in each backcross and three classes in intercrosses, and, thus, Hbb^{d4} segregates as a simple Mendelian trait (Tables 3 and 4). However, the frequency of each phenotypic class was significantly different from 1:1 in three of the four backcrosses; the exception was $Hbb^{s}/Hbb^{s} \times Hbb^{d4}/Hbb^{s}$ (Table 3). In the offspring resulting from backcrosses between heterozygote and homozygous mutant mice there was a deficiency of polycythemic mice Hbb^{d4}/Hbb^{d4}, and in the few offspring of the backcross $Hbb^{d}/Hbb^{d} \times Hbb^{d4}/Hbb^{d}$ there was a deficiency of the heterozygote which was probably due to chance. In the intercrosses the frequencies of the phenotypic classes differed significantly from the expected 1:2:1 ratios, and the deficiency was confined to the homozygous Hbb^{d4}/Hbb^{d4} mice. No deficiencies of the heterozygotes Hbb^{d}/Hbb^{d4} or Hbb^{s}/Hbb^{d4} were found. Increased mortality between birth and weaning (0–18 days) was found for the intercrosses and for those backcrosses involving a polycythemic homozygote Hbb^{d4}/Hbb^{d4} when compared to other mouse stocks and to the backcrosses in which a normal homozygote Hbb^{s}/Hbb^{s} or Hbb^{d}/Hbb^{d} was

TABLE 2

Hematological values

Genotype	No. animals	No. RBC × 10^{-9} ml^{-1} (mean ± SEM)	Hematocrit (%, mean ± SEM)	Hemoglobin ($g \cdot dl^{-1}$, mean ± SEM)	MCV (μm^3, mean ± SEM)	MCHC (%, mean ± SEM)	WBC × 10^{-6} ml^{-1} (mean ± SEM)
Hbb^{d4}/*Hbb*^{d4}	10	14.22 ± 0.78	75.00 ± 0.76	25.43 ± 0.53	54.25 ± 2.42	33.56 ± 0.59	12.34 ± 1.37
Hbb^{d4}/*Hbb*^{d} and *Hbb*^{d4}/*Hbb*^{s}	10	11.09 ± 0.25	55.00 ± 0.42	18.70 ± 0.24	49.80 ± 1.07	34.10 ± 0.50	13.54 ± 1.73
Hbb^{d}/*Hbb*^{d} and *Hbb*^{s}/*Hbb*^{s}	6	9.82 ± 0.32	48.00 ± 0.68	16.22 ± 0.32	49.25 ± 2.04	33.58 ± 0.37	14.18 ± 1.44

TABLE 3

Genotype numbers in polycythemic stock after weaning and losses between birth and weaning

Backcross mating	Birth to weaning loss (%)		Genotype no. after weaning			χ^2 (1 d.f.)
			Hbb^s/Hbb^s	Hbb^s/Hbb^{d4}	Hbb^{d4}/Hbb^{d4}	
$Hbb^s/Hbb^s \times Hbb^{d4}/Hbb^s$	5.4	Obs.	7	8	0	0.066
		Exp.	7.5	7.5	0	$P = 0.80$
$Hbb^{d4}/Hbb^s \times Hbb^{d4}/Hbb^{d4}$	51.9	Obs.	0	16	5	5.76
		Exp.	0	10.5	10.5	$P = 0.016$
			Hbb^d/Hbb^d	Hbb^d/Hbb^{d4}	Hbb^{d4}/Hbb^{d4}	
$Hbb^d/Hbb^d \times Hbb^{d4}/Hbb^d$	2.4	Obs.	15	5	0	5.00
		Exp.	10	10	0	$P = 0.025$
$Hbb^{d4}/Hbb^d \times Hbb^{d4}/Hbb^{d4}$	28.1	Obs.	0	68	38	8.49
		Exp.	0	53	53	$P = 0.0036$

Obs., observed; Exp., expected.

TABLE 4

Genotype numbers in polycythemic stock after weaning and losses between birth and weaning

Intercross mating	Birth to weaning loss (%)		Genotype no. after weaning			χ^2 (2 d.f.)
			Hbb^s/Hbb^s	Hbb^s/Hbb^{d4}	Hbb^{d4}/Hbb^{d4}	
$Hbb^s/Hbb^{d4} \times Hbb^s/Hbb^{d4}$	12.6	Obs.	58	101	19	20.33
		Exp.	44.5	89	44.5	$P = 0.000038$
			Hbb^d/Hbb^d	Hbb^d/Hbb^{d4}	Hbb^{d4}/Hbb^{d4}	
$Hbb^d/Hbb^{d4} \times Hbb^d/Hbb^{d4}$	16.1	Obs.	66	119	35	10.21
		Exp	55	110	55	$P = 0.0061$

Obs., observed; Exp., expected.

used. The loss of mice was even more marked in the backcrosses in which half the offspring were expected to be homozygous mutants than in the intercrosses in which only a fourth of the offspring were expected to be Hbb^{d4}/Hbb^{d4}, and it seems likely that the losses between birth and weaning are mainly of the polycythemic homozyges. Survival of mutant homozygotes after weaning appears to be normal.

It appears unlikely that losses occur before 14 days of gestation since HBB phenotypes were also examined in intercross progeny when they were 14-day-old embryos (day of vaginal plug taken to be day 0) and the frequencies of the phenotypic classes were not significantly different from the expected 1:2:1 ratio (Table 5).

Reciprocal matings of mutant homozygotes with heterozygotes and both

TABLE 5

Genotype Numbers in 14-day embryos

Intercross		Hbb^s/Hbb^s and Hbb^d/Hbb^d	Hbb^s/Hbb^{d4} and Hbb^d/Hbb^{d4}	Hbb^{d4}/Hbb^{d4}
$Hbb^s/Hbb^{d4} \times Hbb^s/Hbb^{d4}$ and $Hbb^d/Hbb^{d4} \times Hbb^d/Hbb^{d4}$	Obs.	15	35	15
	Exp.	16.25	32.5	16.25

Obs., observed; Exp., expected. χ^2 (2 d.f.) = 0.288; P = 0.87.

mutant homozygotes and heterozygotes with the other homozygote were made to ascertain fertility, and a total of 57 matings were set up. Litter size in heterozygous females (6.02 ± 0.22) was not significantly different from normal females (6.61 ± 0.42). None of the nine homozygous mutant females tested produced any litters, although vaginal plugs were observed. Thus, homozygous mutant females appear to be infertile. On the other hand, homozygous mutant males were fertile (mean litter size = 5.31 ± 0.29), and the litter size was similar to that found for heterozygous males (6.04 ± 0.25). Litter size in normal males (9.05 ± 0.67), however, was significantly higher than for both the heterozygous and homozygous mutant males.

Pathology: Five Hbb^{d4}/Hbb^{d4} young adults were killed for pathological observations. At necrospsy, except for the evident plethora, the notable features were splenic enlargement (~300 mg) and cardiac hypertrophy (300–450 mg). In some spleens a few miliary pale patches were observed, and these when visible on X ray were opaque from calcification.

On routine histological examination, in addition to congestion of all tissues to a varying degree, active hematopoietic marrow was found extending throughout the tibia to bones of the tarsus and metatarsus, *i.e.*, peripheral extension. Likewise, the splenic red pulp was grossly hyperplastic with myeloid tissue, mainly erythropoietic; the lymphoid tissue was richly cellular. In two cases the splenic miliary nodules were revealed as trabecular, laminar bone; in other cases in which calcified nodules had been seen by X rays, no bone was seen in the sections available, which may be due to chance or to bone having been ejected by the knife (these spleens were torn during sectioning). Deposition of iron (Perls' stain) in phagocytes was in considerable excess of normal, not only in spleen, mainly red pulp, but in liver and lymph nodes. Perls' stain also revealed hemosiderin in convoluted tubules of the kidneys; this was sometimes visible as brown pigment in routine hematoxylin and eosin sections. Lymph nodes and thymus were of normal architecture, the former perhaps more than usually cellular. Cardiac muscle appeared to be hypertrophic.

Only a few mice were kept for long-term observation. Of four such heterozygotes, one died at 15 months of age but was too autolyzed for examination. One died at 26 months with hemorrhage from an enlarged spleen. One is still alive at 27 months of age, and the original 29-month-old mutant was killed after several weeks of decline; a palpable, enlarged liver was found. A terminal blood count gave much the same figures as formerly [red blood cell (RBC),

12×10^9 ml^{-1}; MCV, 47 μm^3; hemoglobin, 17 g dl^{-1}; MCHC, 30 g dl^{-1}; white blood cell (WBC), 7×10^6 ml^{-1}]. The plasma was jaundiced (3 mg of bilirubin dl^{-1}). At necrospy there was general enlargement of lymph nodes, especially mesenteric, and of liver and spleen, both of which were mottled. The right testicle and epididymis were enlarged and partly cystic. Histology of lymph nodes, thymus, liver and spleen revealed lymphoma (reticulum cell sarcoma of Dunn—a common terminal condition of mice) with multiform cells (lymphoid, histiocytic, eosinophilic polymorphonuclear) and patchy fibrosis; there were many foci with multinucleate giant cells.

Of the homozygotes three are alive, aged between 1 and 2 yr. Two deaths occurred before the age of 1 yr from hemorrhage (gastric and retroperitoneal; three died at 16, 20 and 21 months of age from hemorrhage (ruptured spleen with early lymphoma and portal vein thrombosis, generalized congestion with multiple focal hemorrhages in lungs and multiple internal hemorrhages). Even from a small series one sees that it is the vascular system that is vulnerable.

Physiology: Oxygen dissociation curves were measured for 15 adult mice (seven females, eight males) of the polycythemic stock. Previous investigations of oxygen dissociation curves in inbred strains had revealed no sex differences (NEWTON and PETERS 1983). All of the mice were of the same *Hba* haplotype, Hba^h/Hba^a, since *Hba* is known to affect oxygen affinity (NEWTON and PETERS 1983). The oxygen dissociation curves were measured as described by NEWTON and PETERS (1983) and the mean oxygen equilibrium curves for five polycythemic homozygotes, five heterozygotes Hbb^d/Hbb^{d4} and five normal homozygotes Hbb^d/Hbb^d were determined. The oxygen affinity of the blood of the polycythemic homozygotes was very high, the mean pO_2 at 50% (P_{50}) saturation being 11.4 ± 0.5 mm Hg. The oxygen affinity of the blood of heterozygotes was also increased (P_{50} = 25.8 ± 0.5 mm Hg), but the other homozygote, Hbb^d/Hbb^d, was within the normal range (P_{50} = 46.2 ± 2.0 mm Hg). The oxygen equilibrium curve was hyperbolic in shape for the polycythemic homozygotes and for heterozygotes appeared to be a mixture of the hyperbolic curve and the sigmoidal curve found in normal homozygotes (Figure 2). The value of the Hill coefficient, n, was calculated by the method of least squares. The mean value for the five Hbb^{d4}/Hbb^{d4} mice was 1.80 ± 0.05 and for the normal homozygotes Hbb^d/Hbb^d 2.56 ± 0.07 (Figure 2).

Amino acid analysis: Peptide maps of aminoethylated βd-m1 chain showed all of the expected tryptic peptides with the exception of the C-terminal TyrHis dipeptide which was displaced from its normal position to one more cathodal in electrophoresis and with a lower R_f in chromatography.

Analysis of this abnormal peptide showed that it contained only aminoethylcysteine and histidine, indicating a Tyr → Cys change at position 145 in the mutant βd-m1 chain.

DISCUSSION

The present report is the first of an induced mutation affecting the structure of the major β-globin chain in the mouse. The mutation has resulted in the substitution of tyrosine at β145 by cysteine. Although the β-globin polypeptides

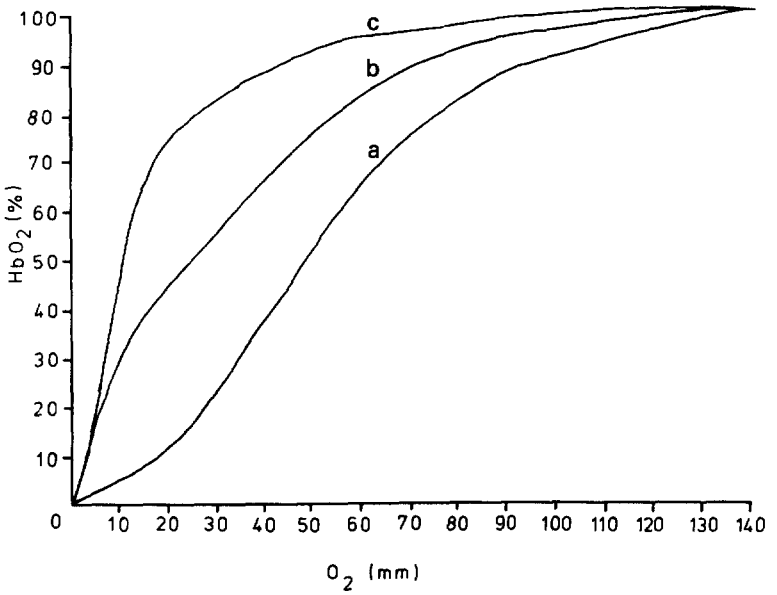

FIGURE 2.—Oxygen equilibrium curves of whole blood from (a) a normal homozygote *Hbb*^{d}/*Hbb*^{d}; (b) a heterozygote *Hbb*^{d}/*Hbb*^{d4} and (c) a polycythemic homozygote *Hbb*^{d4}/*Hbb*^{d4}. The curves were derived using an Aminco Hem-o-scan analyzer at 37°.

of 101/H mice in which the mutation was induced have not been sequenced, other inbred strains, BALB/c, C57BL, SWR and NB, are all known to have tyrosine in this position (POPP 1973; POPP and BAILIFF 1973), and since this tyrosine is one of the few residues common to all known globin sequences of vertebrates (PERUTZ, KENDREW and WATSON 1965), it seems very probable that, in 101/H mice as well, tyrosine is the usual amino acid at position 145. The β145 Tyr → Cys substitution was the only alteration in the βdmaj globin chain that could be found. Tyrosine at β145 in BALB/c and probably also in 101/H is encoded by the TAC codon (KONKEL, MAIZEL and LEDER 1979; VAN OOYEN *et al.* 1979), and the simplest change giving a codon for cysteine is an A to G substitution, thus resulting in TGC. Thus, it is proposed that ENU has induced a G for A substitution in the βdmaj globin gene of the treated 101/H mouse.

This is the second example of single amino acid substitutions in globin polypeptides induced in mouse globin genes by ENU. POPP *et al.* (1983) have also described a germinal mutation induced by ENU in a mouse α-globin gene resulting in the substitution of histidine by leucine at α89. They propose that there has been a single nucleotide substitution from adenine to thymine so that the histidine codon CAC has been replaced by a codon for leucine, CTC. Thus, it seems highly likely that ENU induces small alterations in mammalian genes, but whether single nucleotide substitutions, particularly those involving adenine, are the usual occurrence will not be known until a number of mutants have been analyzed in detail.

When cellulose acetate electrophoresis was carried out on RBC cells lysed in

a dilute solution of EDTA the $\alpha_2\beta$single$_2$, $\alpha_2\beta$dmaj$_2$ and $\alpha_2\beta$d-m1_2 had similar electrophoretic mobilities. WHITNEY (1978) showed that, if mouse hemoglobins are treated with cystamine prior to electrophoresis, the $\alpha_2\beta$single$_2$ has greater anodal electrophoretic mobility than $\alpha_2\beta$dmaj$_2$. Cystamine ($H_2N—CH_2—CH_2—S—S—CH_2—CH_2—NH_2$) reacts with cysteine, adding a half-cystamine to each available cysteine side chain. The addition of a half-cystamine means that a positively charged amino group is added to the protein, resulting in a retarded anodal electrophoretic mobility at alkaline pH compared to an untreated sample. The βsingle polypeptide has one available cysteine at β93, and βdmaj has two, one at β93 and the other at β13 (POPP 1973; POPP and BAILIFF 1973). Thus, the electrophoretic mobility of $\alpha_2\beta$dmaj2 is retarded even more than that of $\alpha_2\beta$single after cystamine treatment. In βd-ml there are three cysteines at β93, 13 and 145, so it might be expected that the $\alpha_2\beta$d-m1_2 would have even lesser anodal electrophoretic mobility than $\alpha_2\beta$dmaj$_2$. Instead, the $\alpha_2\beta$d-m1_2 has a mobility intermediate between $\alpha_2\beta$single$_2$ and $\alpha_2\beta$dmaj$_2$ and, therefore, has greater anodal electrophoretic mobility than $\alpha_2\beta$dmaj$_2$. For hemoglobin Rainier in man, in which there has been also a replacement of tyrosine at β145 by cysteine, GREER and PERUTZ (1971) showed that this new additional cysteine forms a disulfide bridge with the reactive cysteine β93 of the same β-chain. If a similar reaction occurs in the polycythemic mouse, then the number of cysteine side chains available for reaction with cystamine would be reduced. It would not be inconsistent then to find that cystamine treatment modified the electrophoretic mobility of $\alpha_2\beta$d-m1_2 less than would be expected if all of the cysteines were available.

Twenty structurally altered hemoglobins in humans that have high oxygen affinity and lead to polycythemia are listed by MCKUSICK (1983). For 18 of these hemoglobins the structural alteration has occurred in the β-globin polypeptide and for one, hemoglobin Rainier, and substitution is β145 tyrosine $\rightarrow$ cysteine (HAYASHI *et al.* 1971). Thus, hemoglobin Rainier and murine polycythemia, *Hbb*^{d4}, are exact homologues, a rare finding in searches for mouse models of human disorders. Hemoglobin Rainier has increased oxygen affinity, decreased hem-hem interaction and high resistance to alkali denaturation, the alkaline Bohr effect being halved. This abnormal hemoglobin is associated with erythrocytosis and mild rubor (ADAMSON, PARER and STAMATOYANNOPOULOS 1969). Similarly, the hemoglobin of mice of haplotype *Hbb*^{d4} has a high oxygen affinity, decreased hem-hem interactions ($n = 1.80 \pm 0.05$) and leads to polycythemia and rubor, but the alkaline Bohr effect and alkali denaturation have not been investigated yet. GREER and PERUTZ (1971) showed that tyrosine at β145 is essential for hem-hem interaction, and the absence of tyrosine at β145 in hemoglobin Rainier was chiefly responsible for the lack of cooperativity between the globin polypeptides. It seems likely that the consequences of the substitution β145 tyrosine $\rightarrow$ cysteine are the same in the mouse as in man.

Murine polycythemia should be useful in studies of the importance of the difference in oxygen affinity between fetal and maternal blood for a successful outcome of pregnancy. In this context investigations of the cause(s) of infertility in the homozygous mutant female mice may be relevant.

LITERATURE CITED

ADAMSON, J. W., J. J. PARER and G. STAMATOYANNOPOULOS, 1969 Erythrocytosis associated with hemoglobin Rainier: oxygen equilibria and marrow regulation. J. Clin. Invest. **48:** 1376–1386.

BERRY, R. J., 1978 Genetic variation in wild house mice: where natural selection and history meet. Am. Sci. **66:** 52–60.

CLEGG, J. B., M. A. NAUGHTON and D. J. WEATHERALL, 1966 Abnormal human haemoglobins: separation and characterization of the α and β chains by chromatography and the determination of two new variants, Hb Chesapeake and HbJ (Bangkok). J. Mol. Biol. **19:** 91–108.

DAVISSON, M. T. and T. H. RODERICK, 1981 Recombination percentages. pp. 283–313. In: *Genetic Variants and Strains of the Laboratory Mouse*, Edited by M. C. GREEN. Gustav Fischer Verlag, New York.

GILMAN, J. G., 1976a Mouse haemoglobin beta chains. Biochem. J. **155:** 231–241.

GILMAN, J. G., 1976b Mouse haemoglobin beta chains. Biochem. J. **159:** 43–53.

GREER, J. and M. F. PERUTZ, 1971 Three dimensional structure of haemoglobin Rainier. Nature (New Biol.) **230:** 261–267.

HAYASHI, A., G. STAMATOYANNOPOULOS, A. YOSHIDA and J. ADAMSON, 1971 Haemoglobin Rainier, β145 (HC2) tyrosine $\rightarrow$ cysteine and haemoglobin Bethesda: β145 (HC2) tyrosine $\rightarrow$ histidine. Nature (New Biol.) **230:** 264–267.

HILSE, K. and R. A. POPP, 1968 Gene duplication as the basis for amino acid ambiguity in the alpha-chain polypeptides of mouse hemoglobin. Proc. Natl. Acad. Sci. USA **61:** 930–936.

KONKEL, D. A., J. V. MAIZEL and P. LEDER, 1979 The evolution and sequence comparison of two recently diverged mouse chromosomal β-globin genes. Cell **18:** 865–873.

LEDER, A., D. SWAN, F. RUDDLE, P. D'EUSTACHIO and P. LEDER, 1981 Dispersion of α-like globin genes of the mouse to three different chromosomes. Nature **293:** 196–200.

MCKUSICK, V. A., 1983 *Mendelian Inheritance in Man* Ed. 6. The Johns Hopkins University Press, Baltimore.

MUROTA, T., S. SHIBUYA and K. TUTIKAWA, 1982 Genetic analysis of an N-ethyl-N-nitrosourea induced mutation at the hemoglobin β-chain locus in mice. Mutat. Res. **104:** 317–321.

NEWTON, M. F. AND J. PETERS, 1983 Physiological variation of mouse haemoglobins. Proc. R. Soc. Lond. (Biol.) **218:** 443–453.

PERUTZ, M. F., J. C. KENDREW and H. C. WATSON, 1965 Structure and function of haemoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence. J. Mol. Biol. **13:** 669–678.

POPP, R. A., 1973 Sequence of amino acids in the β chain of single hemoglobins from C57BL, SWR and NB mice. Biochim. Biophys. Acta **303:** 52–60

POPP, R. A. and E. G. BAILIFF, 1973 Sequence of amino acids in the major and minor β chains of the diffuse hemoglobin from BALB/c mice. Biochim. Biophys. Acta **303:** 61–67.

POPP, R. A., E. G. BAILIFF, L. C. SKOW, F. M. JOHNSON and S. E. LEWIS, 1983 Analysis of a mouse α-globin gene mutation induced by ethylnitrosourea. Genetics **105:** 157–167.

RUSSELL, E. S. and E. C. MCFARLAND, 1974 Genetics of mouse hemoglobins. Ann. N. Y. Acad. Sci. **241:** 25–38.

RUSSELL, L. B., W. L. RUSSELL, R. A. POPP, C. VAUGHAN and K. B. JACOBSON, 1976 Radiation-induced mutations at mouse hemoglobin loci. Proc. Natl. Acad. Sci. USA **73:** 2843–2846.

RUSSELL, W. L., E. M. KELLY, P. R. HUNSICKER, J. W. BANGHAM, S. C. MADDUX and E. L. PHIPPS, 1979 Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. Proc. Natl. Acad. Sci. USA **76:** 5818–5819.

SKOW, L. C., B. A. BURKHART, F. M. JOHNSON, R. A. POPP, D. M. POPP, S. Z. GOLDBERG, W. F. ANDERSON, L. B. BARNETT and S. E. LEWIS, 1983 A mouse model for β-thalassemia. Cell **34:** 1043–1052.

VAN OOYEN, A., J. VAN DEN BERG, N. MANTEI and C. WEISSMAN, 1979 Comparison of total sequence of a cloned rabbit β-globin gene and its flanking regions with a homologous mouse sequence. Science **266:** 337–344.

WEAVER, S., N. L. HAIGWOOD, C. A. HUTCHINSON, III and M. H. EDGELL, 1979 DNA fragments of the Mus musculus β-globin haplotypes *Hbb*^{s} and *Hbb*^{d}. Proc. Natl. Acad. Sci. USA **76:** 1385–1389.

WHITNEY, J. B., III, 1978 Simplified typing of mouse hemoglobin (*Hbb*) phenotypes using cystamine. Biochem. Genet. **16:** 667–672.

WHITNEY, J. B., III, G. T. COPLAND, L. C. SKOW and E. S. RUSSELL, 1979 Resolution of products of the duplicated hemoglobin α-chain loci by isoelectric focusing. Proc. Natl. Acad. Sci. USA 76: 867–871.

WHITNEY, J. B., III and E. S. RUSSELL, 1980 Linkage of genes for adult α-globin and embryonic α-like globin chains. Proc. Natl. Acad. Sci. USA **77:** 1087–1090.

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